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Isolated Recombinant Domain of von Willebrand Factor Displaying Increased Sensitivity to Ristocetin

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Type 2B von Willebrand disease is characterized by an abnormal von Willebrand factor molecule with increased affinity for the platelet glycoprotein (GP) Ib-IX receptor. A diagnostic feature of type 2B von Willebrand disease is a characteristic loss of von Willebrand factor high molecular weight multimers. In vitro, the soluble interaction of normal von Willebrand factor with platelets can be initiated with exogenous modulators, the most common being the antibiotic ristocetin. The variant molecules resulting in type 2B von Willebrand disease can sustain binding to platelets at subnormal levels of ristocetin. We characterized the von Willebrand factor gene of an individual with type 2B von Willebrand disease and identified a nucleotide transition resulting in an $Arg^{543}\rightarrow Trp$ amino-acid substitution within the GP Ib-IX binding domain of von Willebrand factor. In this study we demonstrate that a recombinant plasmid capable of expressing the isolated GP Ib-IX binding domain of von Willebrand factor, and containing the $Arg^{543}\rightarrow Trp$ amino-acid substitution, secretes a dimeric molecule that supports platelet agglutination using subnormal levels of ristocetin. These results demonstrate that the mutation at position 543 increases the affinity between the variant molecule and platelet GP Ib-IX as an intrinsic feature of the isolated von Willebrand factor domain. Thus, structural perturbations within the GP Ib-IX binding domain that are independent of the von Willebrand factor multimeric structure can sufficiently increase the affinity of von Willebrand factor to sustain platelet aggregation, using subnormal levels of ristocetin.

Key words: type 2B vWD, von Willebrand factor, recombinant fragment

INTRODUCTION

Von Willebrand factor is a multimeric glycoprotein of plasma and the subendothelial matrix that supports two essential processes for normal hemostasis and coagulation [1]. First, it interacts with components of the vessel wall and platelet glycoprotein (GP) receptors to promote platelet adhesion and thrombus formation at sites of vascular injury. Secondly, it serves as a carrier or stabilizing protein for coagulation factor VIII. For supporting platelet attachment, von Willebrand factor has functional domains interacting with the platelet receptor complexes, GP Ib-IX and GP IIb-IIIa (integrin $\alpha_{IIB}\beta_3$) [2]. Current models suggest that the two platelet-binding sites are integrated into a temporal sequence of events initiated by the interaction of subendothelial-bound von Willebrand factor and the platelet GP Ib component of the Ib-IX receptor complex under flow conditions that generate high shear stress [3–9]. This binding event establishes platelet contact with the damaged surface and initiates signals associated with platelet activation [10–12]. Subsequently, von Willebrand factor interacts with the GP IIb-IIIa receptor to establish irreversible adhesion and support platelet aggregation [7–9].

Von Willebrand disease is the most common inherited bleeding disorder in humans and is characterized by a wide variety of subtypes with distinct phenotypic manifestations, each caused by a qualitative and/or quantitative defect in von Willebrand factor [13]. Among the different subtypes, type 2B has attracted considerable attention because type 2B von Willebrand factor has an increased affinity for platelets. In the laboratory, type 2B von Willebrand factor can be identified by its ability to support

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platelet aggregation at subnormal levels of ristocetin [13]. It has been proposed that during thrombogenesis the physiological equivalent of ristocetin may be specific functional conformations acquired by von Willebrand factor or the platelet GP Ib-IX receptor. Such conformational changes might mimic the intrinsic changes within normal von Willebrand factor resulting from binding to subendothelial matrix components, unidentified agonists, or rheological conditions that normally support a platelet-von Willebrand factor interaction [1,14].

In this report, we analyze the von Willebrand factor gene of a type 2B von Willebrand disease patient, leading to the identification of a single point mutation resulting in an amino acid substitution of Arg^{543}→Trp. This previously-reported point mutation was introduced into an expression vector containing the von Willebrand factor coding sequence for amino acids spanning the GP Ib-IX binding domain, Asp^{441}–Asn^{730}. The expressed recombinant fragment was analyzed for its ability to support platelet aggregation in the presence of ristocetin, and the results document the intrinsic ability of an isolated domain to be modulated at low doses of ristocetin. Thus, the recombinant fragment behaves in a manner similar to that of the plasma-derived molecule, demonstrating that the molecular pathology of this type 2B variant is independent of the multimeric structure of von Willebrand factor.

MATERIALS AND METHODS

Molecular Cloning Reagents

T4 DNA ligase, T4 polynucleotide kinase, T7 DNA polymerase and Sequenase version 2.0 were purchased from United States Biochemical (Cleveland, OH). Taq polymerase was purchased from Perkin Elmer Cetus (Norwalk, CT). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA). Nucleotide reagents were obtained from Pharmacia, Inc. (Piscataway, NJ). [^{35}S]dATP was purchased from Amersham (Arlington Heights, IL).

Multimer Analysis

The multimeric structure of plasma von Willebrand factor was analyzed by SDS-agarose gel electrophoresis in a discontinuous buffer system, according to the method described by Ruggeri and Zimmerman [15]. The running gel was composed of 1.6% high-gelling-temperature agarose, and multimers were visualized by autoradiography after incubation with an ^{125}I-labeled rabbit anti-von Willebrand factor antibody.

Polymerase Chain Reaction and DNA Sequencing

Genomic DNA was prepared from peripheral white blood cells using standard procedures [16]. Polymerase chain reaction (PCR) [17] was performed with a Perkin Elmer-Cetus DNA thermal cycler. Five hundred nanograms of human peripheral leukocyte DNA were amplified with 2.5 U of Taq polymerase in a final volume of 100 μl containing 50 pmol of each oligonucleotide primer, as described previously [18]. The primers used for amplification of exon 28 [19] were: 400NG, $^{5'}$ GTC AG*G AAT TCT* GGG AAT ATG GAA GTC ATT G $^{3'}$; and 745NG, $^{5'}$ ACT GA*A AGC TTC* CGA TCC TTC CAG GAC GAA C $^{3'}$. The additional 5′ nucleotides in italics were included to add an *Eco*RI restriction site on 400NG and a *Hin*dIII restriction site on 745NG. The amplified fragments were digested with *Eco*RI and *Hin*dIII, purified with Geneclean glass beads (Bio 101, La Jolla, CA), and subcloned into M13-based vectors for DNA sequence by the method of Sanger et al. [20].

Site-Directed Mutagenesis

We exploited a previously-described recombinant plasmid, pAD5/WT [21,22], that directs the secretion of a von Willebrand factor fragment Arg^{441}–Asn^{730}. The *Xho*I-*Not*I fragment from pAD5/WT, which contains the entire von Willebrand factor-coding sequence for residues 441–730, was subcloned into the polylinker site of pBS/KS^{-}. Uracil-containing single-stranded template was generated using *Escherichia coli* CJ236 for site-directed mutagenesis [23]. After introducing the mutation, resulting in an Arg^{543}→Trp substitution, the plasmid was fully sequenced to confirm that it contained the desired codon substitution without other spontaneous errors. The *Xho*I-*Not*I fragment with codon substitution was then subcloned into a mammalian expression vector with a neomycin-resistant gene, pcDM8neo [21], and designated "pAD5/R543W."

Cell Culture and Transfection

Chinese hamster ovary (CHO-K1) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and subcultured twice a week. Mutated recombinant plasmid, pAD5/R543W, was isolated by the procedure of Birnboim and Doly [24] and purified in cesium chloride gradients generated by ultracentrifugation [25]. Transfection of CHO-K1 cells was performed using 5 μg of pAD5/R543W and a calcium-phosphate procedure [26]. Stable transformants were selected in the aminoglycoside antibiotic, G418 (Geneticin; Sigma, St. Louis, MO), and identified by screening the culture media with the anti-von Willebrand factor monoclonal antibody, LJ-RG46 [27].

Platelet Agglutination Assay

Serum-free conditioned media of stable CHO-K1 transformants were dialyzed against HEPES-buffered saline (20 mM HEPES, pH 7.4, 150 mM NaCl) and concentrated by centrifugation through Centricon 30 filters (Amicon, Beverly, MA). The amount of von Willebrand factor antigen was normalized by the intensity of immunoreactivity with the anti-von Willebrand factor monoclonal antibody, LJ-RG46 [28]. Platelet aggregation was examined in sili-

TABLE I. Laboratory Data of Patient With Type 2B von Willebrand Disease

Bleeding time (min)	FVIII:C (%)	vWF:Ag (%)	vWF:Rcof (%)
>10	14	25	61

conized glass cuvettes in a dual-channel aggregometer (Chrono-Log Corp.). Aggregation was recorded as an increase of light transmittance through the stirred platelet suspension. Washed platelets were prepared by the previously described method [29], and used at a final concentration of 1×10^8/ml in the presence of ristocetin.

RESULTS

Patient Profile

A 47-year-old female with bleeding history was referred to the Department of Clinical Laboratory Medicine, Toyama Medical and Pharmaceutical University, in December 1991. Laboratory examinations confirmed decreased coagulation factor VIII activity, reduction in both vWF:Ag and vWF:Rcof, increased ristocetin-induced platelet aggregation (Table I), and an absence of the largest von Willebrand factor multimers in plasma (Fig. 1). Platelet aggregation of patient plasma was observed with as little as 0.3 mg/ml of ristocetin, whereas normal plasma required at least 0.6 mg/ml of ristocetin. These data formed the basis for establishing a type 2B von Willebrand disease phenotype [13]. Informed consent was obtained from the patient before conducting our study, in accordance with the Declaration of Helsinki.

Amplification and Characterization of von Willebrand Factor Exon 28 Sequence

A portion of exon 28 was amplified from the propositus' genomic DNA. Conditions that permitted the specific amplification of the von Willebrand factor gene sequence without coamplification of the pseudogene were used [19]. Amplification yielded a fragment of 958 base pairs (bp), which contained the coding sequence for amino-acid residues 463–743 of mature von Willebrand factor. After restriction enzyme digest, the amplified fragments were subcloned into M13-based vectors and their DNA sequence determined. A single nucleotide transition, C→T, was observed in 6 of 9 clones within codon 543, suggesting that the patient had one allele with a normal Arg^{543} codon (CGG) and a second allele containing a Trp^{543} codon (TGG), resulting in an Arg^{543}→Trp substitution (Fig. 2). An Arg^{543}→Trp substitution is one of several amino acid substitutions which have been shown to result in the type 2B von Willebrand factor phenotype [30]. An additional codon substitution, Ala^{618}→Thr, was also observed and is a known polymorphism of von Willebrand factor (data not shown) [31].

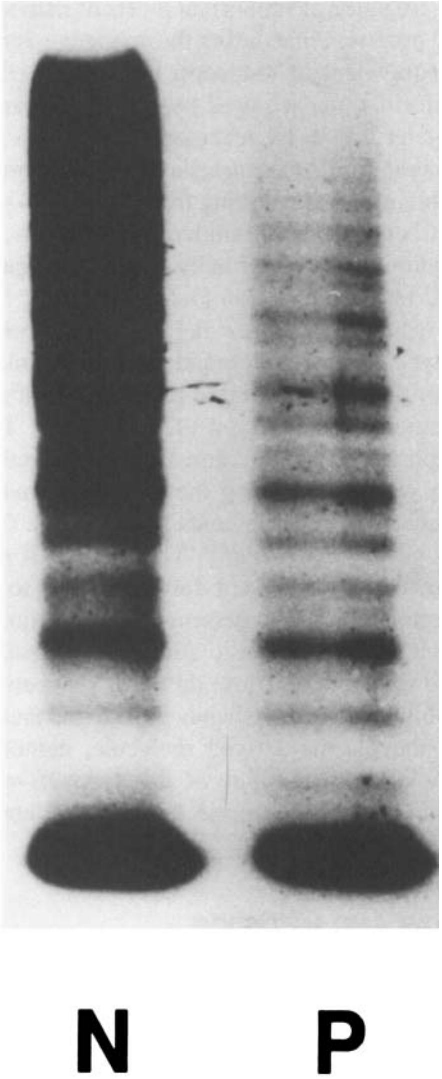

Fig. 1. Multimeric structure of von Willebrand factor in normal (N) and patient (P) plasma. SDS-agarose gel electrophoresis was performed in an intermediate resolution gel (1.6% agarose). Von Willebrand factor was visualized by autoradiography after treating the gel with an iodinated rabbit anti-human von Willebrand factor polyclonal antibody.

Expression and Functional Analyses of the Expressed Molecules

Recombinant proteins with an Arg^{543}→Trp substitution (designated "r116/Trp^{543}") and normal von Willebrand factor sequence (r116/WT) were expressed as isolated domains in CHO-K1 cells. Both the wild-type and the mutant protein migrated with the same electrophoretic pattern, consisting of a similar mixture of dimer and monomer (Fig. 3A). The culture media containing the fragments were dialyzed against HEPES-buffered saline and concentrated up to 200-fold by centrifugation. Platelet aggregation assays were performed with both proteins in the presence of various concentrations of ristocetin (Fig.

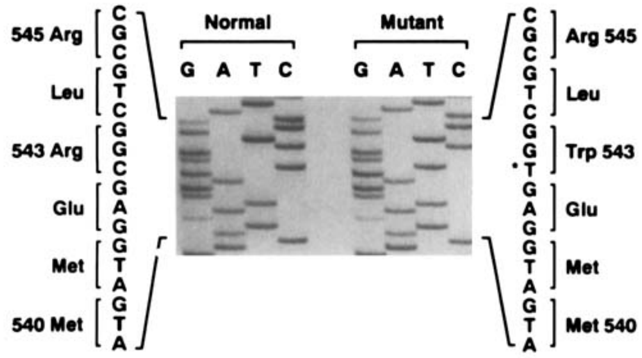

Fig. 2. Autoradiograph illustrating DNA sequence analysis of patient's mutation as compared to a similar sample containing normal von Willebrand factor DNA sequence. Asterisk indicates C→T transition within codon 543, resulting in an Arg→Trp mutation.

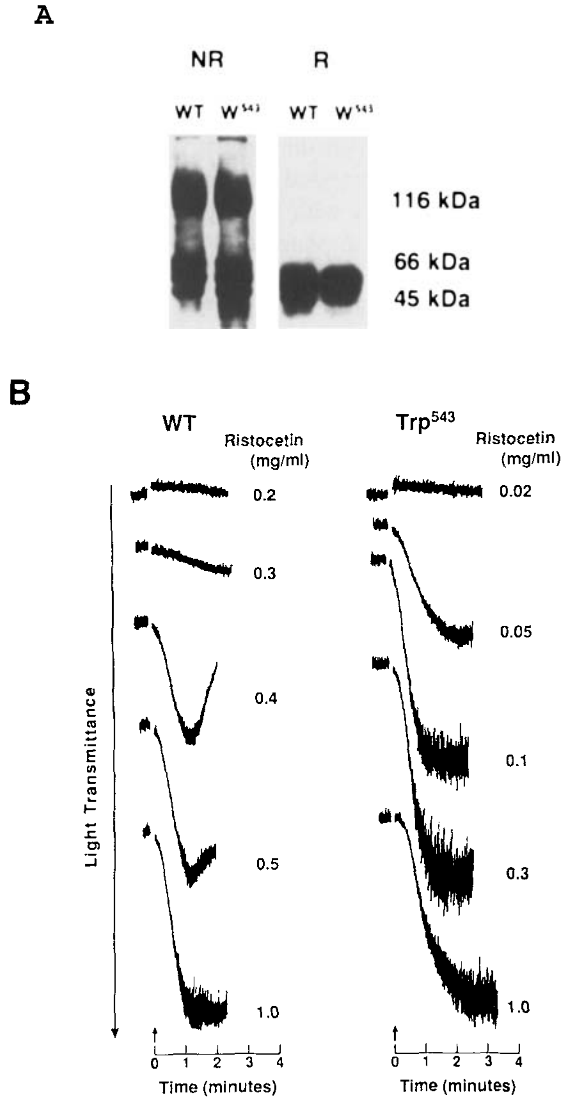

Fig. 3. A: Concentrated culture media from CHO-K1 cells, secreting wild-type (WT) and mutant (W543) proteins, were subjected to electrophoresis in 10% SDS-PAGE. Samples were electrophoresed under nonreducing (NR) and reducing (R) conditions. Proteins were visualized after immunoblotting to nitrocellulose, reacting with the anti-von Willebrand factor monoclonal antibodies, LJ-RG46 and LJ-52-K2 [27], and an iodinated rabbit anti-mouse IgG. Corresponding migration of molecular standards is shown at right. B: Concentrated culture media samples were incubated with washed platelets in an aggregometer cuvette. Ristocetin at indicated final concentrations were added at positions of arrows. Extent of aggregation is monitored as a change in light transmittance.

3B). The r116/WT molecules supported platelet aggregation at a ristocetin concentration as low as 0.4 mg/ml, while r116/Trp^{543} supported aggregation with as little as 0.05 mg/ml of ristocetin. No platelet aggregation was observed in the absence of ristocetin or using conditioned medium derived from CHO-K1 cells transfected with the expression vector pcDM8neo, as previously reported [21,22,28].

DISCUSSION

The in vivo modulation of von Willebrand factor binding to GP Ib-IX poses a central problem in understanding the molecular bases of hemostasis, and can be illustrated by the observation that soluble plasma von Willebrand factor has an unmeasurable affinity for GP Ib-IX, and yet under rheological conditions producing high-shear stress, von Willebrand factor and GP Ib-IX create an essential ligand-and-receptor interaction that supports platelet adhesion and activation. One crucial molecular structure of von Willebrand factor that influences its adhesive properties is the formation of von Willebrand factor multimeric complexes (multimers). Indeed, a direct relationship exists between increasing multimer size and higher potency in platelet adhesion [32]. Although multimer formation is essential for von Willebrand factor in vivo activity, it is becoming increasingly apparent that the native conformation of the GP Ib-IX binding domain of the von Willebrand factor subunit intrinsically regulates its affinity for platelets [33,34].

We analyzed the DNA sequence of a type 2B von Willebrand disease patient and found a single point mutation resulting in an Arg^{543}→Trp amino-acid substitution. To date, there are 11 mutations that have been associated with the type 2B phenotype, and 8 of the 11 mutations are within an intramolecular disulfide loop formed between Cys^{509}–Cys^{695} [30]. The phenotypic consequences of the Arg^{543}→Trp mutation were evaluated using a recombinant plasmid that expresses in heterologous cells an isolated von Willebrand factor domain [21,22]. The expressed polypeptide, composed of von Willebrand factor residues 441–730, was capable of secretion and dimerization, irrespective of an Arg or Trp at position 543. However, in

functional assays, the molecule containing an $Arg^{543}{\rightarrow}Trp$ amino-acid substitution supported platelet aggregation in the presence of ristocetin at 0.05 mg/ml, while wild-type molecules required at least 0.4 mg/ml of ristocetin. This observation agrees with the results reported by Cooney et al. [35], who expressed full-length von Willebrand factor in COS-7 cells with an $Arg^{543}{\rightarrow}Trp$ and observed an increased affinity to platelet GP Ib-IX in the presence of subnormal levels of ristocetin. This latter observation provided definitive proof that an $Arg^{543}{\rightarrow}Trp$ amino-acid substitution can result in the clinical phenotype, designated "type 2B von Willebrand disease."

In previous studies, we focused on the coding sequence for the GP Ib-IX binding domain within a type 2B von Willebrand factor molecule and identified a $Trp^{550}{\rightarrow}Cys$ substitution [33]. By expressing the isolated domain in prokaryotic and eukaryotic cells, it became apparent that the native conformation of normal von Willebrand factor prevents binding of soluble von Willebrand factor to platelet GP Ib-IX. This conclusion was based on the observation that recombinant molecules expressed in eukaryotic cells, such as Chinese hamster ovary cells, acquired a disulfide bond-dependent conformation that is analogous to plasma von Willebrand factor and, like soluble von Willebrand factor, requires ristocetin to bind platelet GP Ib-IX [21,22]. When the same recombinant domain was expressed containing a $Trp^{550}{\rightarrow}Cys$ amino-acid change, the mutant domain spontaneously interacted with platelet GP Ib-IX in binding studies, but failed to support platelet aggregation [33]. Thus, in the previous characterization of the type 2B von Willebrand factor molecule [33], the new Cys residue at position 550 presumably disrupted the disulfide bond-dependent conformation, yielding a molecule that can bind to platelet GP Ib-IX, but that is unable to support platelet aggregation. In support of this explanation, the formation of the intramolecular disulfide loop between Cys^{509}–Cys^{695} is essential to generate a molecule that can support ristocetin-mediated platelet aggregation [22].

In contrast, the present study illustrates with a different amino-acid substitution ($Arg^{543}{\rightarrow}Trp$) that the ability to support platelet aggregation at subnormal levels of ristocetin can be mimicked with a dimeric domain of von Willebrand factor. Thus, the clinical observation of increased platelet agglutination at lower concentrations of ristocetin can be mimicked in a dimeric fragment of von Willebrand factor, illustrating that structural perturbations within the isolated domain is responsible for these clinical observations.

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